Mutation of *kvrA* causes OmpK35/36 porin downregulation and reduced meropenem/vaborbactam susceptibility in KPC-producing *Klebsiella pneumoniae*.

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P.D. finished the work for publication and so is named as first author.

Running Title: KvrA loss reduces porin production in *K. pneumoniae*

Abstract

Meropenem/vaborbactam resistance in *Klebsiella pneumoniae* is associated with loss of function mutations in the OmpK35 and OmpK36 porins. Here we identify two previously unknown loss of function mutations that confer cefuroxime resistance in *K. pneumoniae*. The proteins lost were NlpD and KvrA; the latter is a transcriptional repressor controlling capsule production. We demonstrate that KvrA loss reduces OmpK35 and OmpK36 porin production, which confers reduced susceptibility to meropenem/vaborbactam in a KPC-3 producing *K. pneumoniae* isolate.

Text

Carbapenems are often reserved as a last resort for treatment of severe infections caused by multi-drug resistant Gram-negative bacteria. A rise in the prevalence of cephalosporin resistance, particularly due to the spread of mobile cephalosporinase genes in the Enterobacteriaceae has resulted in the increased use of carbapenems worldwide. This has driven the emergence of carbapenem resistant Enterobacteriaceae (CRE) which are classed as one of the greatest threats to human health according to the World Health Organisation. In *Klebsiella pneumoniae,* carbapenem resistance is mainly mediated by the production of a carbapenemase. In some parts of the world the most prevalent is the class A carbapenemase KPC; in others, most common are the class B metallo-β-lactamases e.g. NDM; in others, class D carbapenemases e.g. OXA-48 like enzymes predominate (1-3). CRE can also emerge due to mutations that reduce envelope permeability, for example those that result in porin deficiency, and particularly when these mutations occur in isolates producing CTX-M or AmpC-type cephalosporinases (4). Indeed, most carbapenem resistant *K. pneumoniae* clinical isolates have multiple resistance mechanisms, including permeability defects (5-8).

As part of our work aiming to identify novel mechanisms for reduced cephalosporin and/or carbapenem permeability in *K. pneumoniae*, the *oqxR* and *ramR* double mutant, FQ3, derived from *K. pneumoniae* Ecl8 as previously described (9) was used as a parent strain to select cephalosporin resistant mutants. FQ3 overproduces two efflux pumps; AcrAB and OqxAB, resulting in resistance to fluoroquinolones, chloramphenicol and minocycline (9). One hundred microlitre aliquots of overnight cultures grown in Nutrient Broth (NB) were spread onto Mueller Hinton Agar containing 16 $\mu g.mL^{-1}$ cefuroxime, which were then incubated for 24 h. One representative mutant derivative was named “FQ3 M1” and was shown by disc susceptibility testing, performed and interpreted according to CLSI methodology (10, 11) to be resistant to cefuroxime, cefoxitin and cephalexin (**Table 1**). MICs were determined for a selected group of antimicrobials, confirming cefuroxime and cefoxitin resistance, and revealing small increases in the MICs of third generation cephalosporins and aztreonam against FQ3 M1, but these remained in susceptible ranges (**Table 1**).

Changes in envelope protein abundance in mutant FQ3 M1 relative to FQ3 were quantified using LC-MS/MS proteomics as previously described (12) to identify potential reasons for cephalosporin resistance. There were 15 proteins significantly altered (**Table 2**). Amongst this group of proteins with uncertain or metabolic functions, were the important antimicrobial entry porins OmpK35 and OmpK36, which were both downregulated in FQ3 M1 by almost 3-fold.

We next used WGS, aiming to explain the downregulation of OmpK35 and OmpK36 in mutant FQ3 M1 relative to FQ3. WGS was performed by MicrobesNG (Birmingham, UK) using a

HiSeq 2500 instrument (Illumina, San Diego, CA, USA). Reads were trimmed using Trimmomatic (13) and assembled into contigs using SPAdes 3.10.1 (http://cab.spbu.ru/software/spades/). Assembled contigs were mapped to the *K. pneumoniae* Ecl8 reference genome (GenBank accession number GCF_000315385.1) by using progressive Mauve alignment software (14).

No mutations were detected in *ompK36* and *ompK35* or adjacent sequences, or in known regulators of porin production, e.g. OmpR/EnvZ (15). In fact, FQ3 M1 has three separate mutations relative to FQ3: a frameshift mutation (causing Asn278FS) in *nlpD*, a frameshift (causing Asp395FS) in *dhaR*, and a 1,159 bp deletion spanning *kvrA* and the adjacent genes, *ydhI* and *ydhJ*. In *Escherichia coli,* NlpD is involved in peptidoglycan remodelling during cell division (16-18). DhaR is a transcriptional activator responsible for controlling the production of the metabolic enzymes glycerol dehydrogenase and dihydroxyacetone kinase (19,20). KvrA is a MarR-type transcriptional repressor with a key role in *K. pneumoniae* capsulation (21-23).

In order to deconvolute the possible roles of the three different loss of function mutations, we separately insertionally inactivated *nlpD*, *dhaR*, *kvrA*, and also *ompK36* as controls in FQ3. Mutants were constructed using the pKNOCK suicide plasmid (24). DNA fragments of the genes to be inactivated were amplified with Phusion High-Fidelity DNA Polymerase (NEB, UK) from *K. pneumoniae* Ecl8 genomic DNA by using primers *ompK36* KO FW (5′-CGTTCAGGCGAACAACACTG-3′) and *ompK36* KO RV (5′-AAGTTCAGGCCGTCAACCAG-3′); *kvrA* KO FW (5’-ATCTGGCACGTTTAGTTCGC-3’) and *kvrA* KO RV (5’-CCCTTTCTCCTCCAGCTGAT-3’); *dhaR* KO FW (5’-CAATCAGATGTACGGCCTGC-3’) and *dhaR* KO RV (5’-GACTTCGACGTGATTCAGGC-3’); *nlpD* KO FW (5’-ACGATTTCCGCGACCTGGCG-3’) and *nlpD* KO RV (5’- CAACATCTTGGTAGCACTCT-3’). Each PCR product was separately ligated into the pKNOCK-GM at the SmaI site and each recombinant plasmid was then transferred into FQ3 cells by conjugation from *E. coli* BW20767. Mutants were selected using gentamicin (5 $\mu g.mL^{-1}$) and the mutations were confirmed by PCR using primers *ompK36* full length FW (5’-GAGGCATCCGGTTGAAATAG-3’) and *ompK36* full length RV (5’-ATTAATCGAGGCTCCTCTTAC-3’); *kvrA* full length FW (5’-ACTTAGCAAGCTAATTATAAGGAGATGA-3’) and *kvrA* full length RV (5’-GCCGCAAAGAATTAATCTTTA-3’); *dhaR* full length FW (5’- CAGCCCGATGGACGAGATT-3’) and *dhaR* full length RV (5’- TATTGGGCTCAGCGCGTCC-3’); *nlpD* full length FW (5’-GTCGGCGAAGAGCATCAGT-3’) and *nlpD* full length RV (5’-CACCTTCCACGGCACATCA-3’).

Inactivating *dhaR* in FQ3 had no effect on cephalosporin MIC, determined using CLSI methodology (25) but inactivating *nlpD* or *kvrA*, raised cefoxitin and cefuroxime MIC, though

in both cases the MIC was one doubling dilution lower than against FQ3 M1 (**Table 3**). A similar effect was observed upon disruption of *ompK36*, adding further weight to the hypothesis that porin downregulation is involved in the phenotype acquired by FQ3 M1. Whilst *ompK35* disruption did not affect cephalosporin MICs, FQ3 already has downregulated OmpK35 production due to mutations in *ramR* and *oqxR* (9). We next complemented *kvrA* in FQ3 M1. To do this, *kvrA* DNA was amplified from *K. pneumoniae* Ecl8 genomic DNA by using primers (introduced restriction sites underlined): *kvrA* full length BamHI FW (5'-AAAGGATCCCGGCAATCCGGATGTGTTAAGAC-3') and *kvrA* full length SalI RV (5'-AAAGTCGACGGAGGGTGAAAAAAGGCCCGGATTA-3'). The PCR product was digested and inserted to pUBYT (26) cut with BamHI and SalI to generate pUBYT::*kvrA*. The recombinant plasmid was then transferred into FQ3 M1 cells by electroporation. The transformants were selected using kanamycin (50 $\mu g.mL^{-1}$) and the presence of plasmids was confirmed by PCR using primers pUBYT check FW (5'-GCAAGAAGGTGATGAATCTACA-3') and pUBYT check RV (5'-GTGGCAGCAGCCAACTCA-3'). Complementation of FQ3 M1 with pUBYT::*kvrA* showed that the MICs of cefoxitin and cefuroxime reduced, but again not to a value as low as against FQ3. This confirmed the result of the gene inactivation experiment that *kvrA* loss alone is not the sole determinant of the cephalosporin resistant phenotype expressed by FQ3 M1 (**Table 3**).

Given that inactivation of either *kvrA* or *nlpD* in FQ3 reduced cephalosporin susceptibility, we performed LC-MS/MS envelope proteomics for these mutants versus FQ3 as above, which revealed that mutation in *kvrA* caused a reduction in OmpK35 and OmpK36 porin levels in FQ3, to the same extent as seen in FQ3 M1. Mutation in *nlpD*, despite altering cephalosporin MIC, did not (**Fig. 1 A, B**). Of the proteomic changes, in addition to OmpK35 and OmpK36, seen in FQ3 M1 versus FQ3, only 4/13 proteins were similarly up/down regulated upon disruption of *kvrA* in FQ3 (**Table 2**). Because FQ3 is derived from Ecl8, a laboratory strain, and because FQ3 carries mutations that increase efflux pump and reduce OmpK35 porin production (9), we wanted to test the impact of *kvrA* inactivation in a wild-type clinical isolate. To do this, we insertionally inactivated *kvrA* (as above) in the susceptible *K. pneumoniae* clinical isolate KP47, which has wild-type envelope permeability (27) and showed by LC-MS/MS that OmpK35 and OmpK36 levels fell in this mutant relative to KP47, approximately 3-fold, as seen in FQ3. Porin abundance did not change upon *nlpD* inactivation in KP47, as also seen in FQ3 (**Fig. 1 C, D**).

β-Lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam have been successful in overcoming resistance to penicillin derivatives, e.g. amoxicillin, piperacillin and ticarcillin in Enterobacteriaceae. However, inhibitor/penicillin combinations are not clinically useful against KPC, CTX-M, OXA-48-like or AmpC producing Enterobacteriaceae isolates, or

those producing metallo-β-lactamases (28). This has led to the development of new β-lactam/β-lactamase inhibitor combinations, and one recently licenced for clinical use is meropenem/vaborbactam (29-32).).

Meropenem/vaborbactam resistance in KPC-producing *K. pneumoniae* has been shown to occur by loss of function mutation in *ompK35* and *ompK36* (33,34). We therefore wanted to test the impact of OmpK35/36 porin reduction seen following inactivation of *kvrA* in *K. pneumoniae* KP47 (**Fig. 1C**) on meropenem/vaborbactam susceptibility when the mutant produces KPC. To investigate this, we used pUBYT::*bla*$_{KPC-3}$ (26) to transform *K. pneumoniae* KP47, KP47Δ*kvrA* and KP47Δ*ompK36* (as a control). As expected, due to carriage of KPC, all transformants were resistant to meropenem. Addition of vaborbactam (8 μg.mL^{-1}) reduced the meropenem MIC against KP47 well into the susceptible range. In contrast, meropenem/vaborbactam MICs against the *kvrA* and *ompK36* mutants were 1 μg.mL^{-1} (four doubling dilutions higher than against KP47) and 32 μg.mL^{-1} respectively, due to the production of KPC. The meropenem/vaborbactam MIC against FQ3 Δ*kvrA*::*bla*$_{KPC-3}$ was also raised by 3 doubling dilutions to become 1 μg.mL^{-1}. Disruption of *nlpD* in FQ3 or KP47 did not significantly raise meropenem/vaborbactam MIC (**Table 4**) as expected because it has no effect on porin abundance (**Fig. 1**). Hence, even in an otherwise wild-type (27) KPC-producing clinical *K. pneumoniae* isolate, OmpK35/36 downregulation caused solely by *kvrA* mutation is enough to reduce meropenem/varborbactam susceptibility. There was no change in susceptibility to ciprofloxacin, minocycline or chloramphenicol in KP47 Δ*kvrA* or KP47 Δ*nlpD* relative to KP47, confirming that the effects of these mutations are β-lactam focussed. We cannot say for other classes because resistance is caused by acquired mechanisms.

NlpD ("new lipoprotein D") is conserved across Gram-negative bacteria, with essential roles in virulence, e.g. in *Yersinia pestis* (35). In *E. coli* it is recruited to the division site where it targets the activity of the peptidoglycan amidase AmiC, to which it binds. Loss of NlpD in *E. coli* is known to delay the onset of cell lysis after treatment with ampicillin because peptidoglycan breakdown by AmiC is less targeted to the division site (36,37). This provides a clear rationale for why disruption of *nlpD* reduces β-lactam susceptibility in *K. pneumoniae* (**Table 1**), but its role as a mediator of cefuroxime resistance has not previously been suspected.

We also report that inactivation of *kvrA* causes cefuroxime resistance in *K. pneumoniae*, but more importantly, it causes reduced susceptibility to meropenem/vaborbactam, even in an otherwise wild-type *K. pneumoniae* clinical isolate transformed to express *bla*$_{KPC-3}$ from its native promoter in a low copy number vector (26). We show that cefuroxime resistance and reduced meropenem/vaborbactam susceptibility in a *kvrA* mutant is associated with OmpK35

and OmpK36 downregulation. This is reminiscent of OmpR mutations in *E. coli*, which reduce OmpF and OmpC production and can also affect antimicrobial susceptibility (38).

KvrA is a MarR-family transcriptional repressor. Importantly, we found that YdhJ is upregulated 45-fold (p=0.002, n=3) and >100-fold (p<0.0001, n=3) according to proteomics following disruption of *kvrA* in *K. pneumoniae* KP47 and FQ3, respectively. YdhJ is encoded within a putative efflux pump operon adjacent to *kvrA* on the chromosome. Expression of the homologue of this *ydhIJK* operon in *E. coli* is directly repressed by SlyA (39), which is encoded upstream of *ydhIJK*, in an almost identical arrangement as *kvrA* and *ydhIJK* in *K. pneumoniae*. Therefore, the *ydhIJK* operon is likely to be the direct repressive target of KvrA in this species, with its wider activatory effects being indirect. Similar characterised MarR-family repressors such as SlyA also tend to have local direct repressive effects, but cause activation of gene expression at some promoters indirectly by blocking the repressive activity of H-NS at those promoters (40). Since H-NS is known to have a repressive effect on porin production in *E. coli* (41) it may well be that increased repressive activity of H-NS in the absence of KvrA is the explanation for OmpK35/36 downregulation seen in *K. pneumoniae* following loss of function mutation in *kvrA*.

Disruption of *kvrA* also causes downregulation of key capsule biosynthesis genes, e.g. *galF*, *manC* and *wzi* in some *K. pneumoniae* strains (21). We found using proteomics that Wzi was downregulated a marginal 0.82-fold (p=0.03, n=3) upon disruption of *kvrA* in isolate KP47 but not GalF or ManC and none of these proteins were downregulated in FQ3 Δ*kvrA* versus FQ3. However, it is known that *kvrA* loss does not reduce capsule production is all strains, and nor does it attenuate the virulence of all strains in a mouse infection model (21). Among 455 isolates of *K. pneumoniae* from NCBI database, we found 9 isolates (Genbank accession numbers CP037927, LR134162, CP032175, CP018056, CP003200, LR588409, CP044039, CP043670, CP043669) where *kvrA* was mutated in a way predicted to result in a truncated and presumably non-functional KvrA. One key example is the KPC-2 and CTX-M-14 producing carbapenem resistant human sputum isolate HS11286 (42, 43). This confirms that *kvrA* mutants are present in human clinical samples.

In conclusion, we report here two loss of function mutations in genes previously not known to affect antimicrobial susceptibility in *K. pneumoniae*. Both cause early generation cephalosporin resistance, using two different mechanisms. The mechanism stimulated by *kvrA* loss is reduced porin production, and, importantly, this reduces susceptibility to one of the most recently released β-lactam/β-lactamase inhibitor combinations.

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We declare no conflicts of interest.

Figure Legends

Figure 1. Normalised abundance of OmpK35 and OmpK36 porins in *K. pneumoniae kvrA* and *nlpD* mutants.

OmpK36 and OmpK35 abundances were measured using LC-MS/MS and normalised to the abundance of OmpA, to control for protein loading. Data presents mean ± standard error of the mean, n=3. Asterisk (*) indicates statistically significant differences from the parental strains by t-test (p<0.05). (**A**) OmpK36 to OmpA protein ratio and (**B**) OmpK35 to OmpA protein ratio in FQ3 and its mutant derivatives. (**C**) OmpK36 to OmpA protein ratio and (**D**) OmpK35 to OmpA protein ratio in clinical isolate KP47 and its mutant derivatives.

Tables

Table 1. Susceptibility testing for antimicrobials against *K. pneumoniae* FQ3 and cefuroxime resistant mutant FQ3 M1.

Antimicrobial	Parental strain, FQ3 Disc Test	MIC	FQ3 M1 Disc Test	MIC
Meropenem	S	0.125	S	0.125
Ertapenem	S		S	
Imipenem	S		S	
Doripenem	S		S	
Aztreonam	S	0.0625	S	0.125
Cefepime	S		S	
Ceftazidime	S	0.125	S	0.25
Cefotaxime	S	0.25	S	1
Ceftriaxone	S		S	
Ceftizoxime	S		S	
Cefoperazone	S		S	
Cefotetan	S		S	
Cefoxitin	S	8	R	64
Cefuroxime	S	8	R	64
Cephalexin	S		R	
Ciprofloxacin	R	8	R	8
Norfloxacin	R		R	
Ofloxacin	R		R	
Levofloxacin	R		R	
Chloramphenicol	R	128	R	128
Minocycline	R	16	R	16
Amikacin	S	1	S	2
Gentamicin	S		S	

Susceptibility (S) or resistance (R, shaded) was defined using CLSI breakpoints (11).

Table 2. Significant changes in envelope protein abundance seen in *K. pneumoniae* mutant FQ3 M1 versus parent strain FQ3

Accession	Description	Abundance FQ3 (1)	Abundance FQ3 (2)	Abundance FQ3 (3)	Abundance FQ3 M1 (1)	Abundance FQ3 M1 (2)	Abundance FQ3 M1 (3)	T-test	Fold change
A6T518	Phosphoheptose isomerase, GmhA	-	-	-	1.18E+08	2.05E+07	2.02E+07	<0.0005	>20
A6T720	Aspartate aminotransferase, AspC	-	-	-	2.11E+07	1.92E+07	2.27E+07	<0.0005	>20
A6TBN4	ATP-binding component of methyl-galactoside transporter, MglA	-	-	-	2.64E+07	2.11E+07	3.40E+07	<0.0005	>20
A6TF45	sn-glycerol-3-phosphate dehydrogenase, GlpD	3.10E+07	1.21E+07	3.24E+07	2.37E+08	1.24E+09	1.72E+09	0.004	42.43
A6T5Q5	Putative outer membrane protein	4.62E+08	3.06E+08	4.48E+08	1.61E+09	1.85E+09	1.51E+09	<0.0005	4.09
A6TBM3	Putative channel/filament proteins, YohG	2.93E+08	2.57E+08	4.77E+08	9.04E+08	1.04E+09	1.31E+09	<0.0005	3.16
A6T4Y7	Acetyl-coenzyme A carboxylase carboxyl transferase, AccA	1.24E+08	8.31E+08	4.06E+08	1.15E+09	7.38E+08	9.36E+08	0.005	2.07
A6T8N5	Putative uncharacterized protein, YdgH	6.65E+08	5.22E+08	8.53E+08	3.69E+08	3.61E+08	3.44E+08	0.001	0.53
A6T721	OmpK35 porin	1.19E+10	7.32E+09	7.82E+09	2.89E+09	3.97E+09	3.01E+09	0.001	0.36
A6TBT2	OmpK36 porin	3.49E+10	3.13E+10	3.41E+10	1.12E+10	1.31E+10	1.00E+10	<0.0005	0.34
A6TD34	Lipoprotein, NlpD	9.15E+08	7.51E+08	1.14E+09	2.64E+08	1.68E+08	3.13E+08	<0.0005	0.27
A6T4Y4	Lipid-A-disaccharide synthase, LpxB	6.06E+07	5.19E+07	1.02E+08	-	-	-	<0.0005	<0.005
A6T9Z3	Putative multidrug resistance protein, YdhJ	7.23E+07	6.50E+07	9.18E+07	-	-	-	<0.0005	<0.005
A6TBM5	Putative cellobiose-specific PTS permease	2.83E+07	1.66E+07	3.57E+07	-	-	-	<0.0005	<0.005
A6TEA5	Putative glycerol dehydrogenase, DhaD	5.00E+07	2.28E+08	2.51E+08	-	-	-	<0.0005	<0.005

Strains were grown in Cation-Adjusted Muller-Hinton broth and raw abundance data are provided for three biological replicates of parent (FQ3) and mutant (FQ3 M1). Analysis was as described in (9) and proteins listed are those with significantly different abundance in FQ3 M1 versus FQ3. Proteins with accession numbers highlighted in bold/underline were similarly up/down regulated in FQ3 Δ*kvrA* relative to FQ3.

Table 3. MICs (µg.mL^{-1}) of cephalosporins against *K. pneumoniae* Ecl8 FQ3 and mutant derivatives.

	Cefuroxime MIC	**Cefoxitin MIC**
FQ3	8	8
FQ3 Δ*dhaR*	8	8
FQ3 Δ*ompK35*	8	8
FQ3 Δ*kvrA*	16	32
FQ3 Δ*nlpD*	32	32
FQ3 Δ*ompK36*	32	32
FQ3 M1	64	64
FQ3 M1(pUBYT)	32	64
FQ3 M1(pUBYT::*kvrA*)	16	16

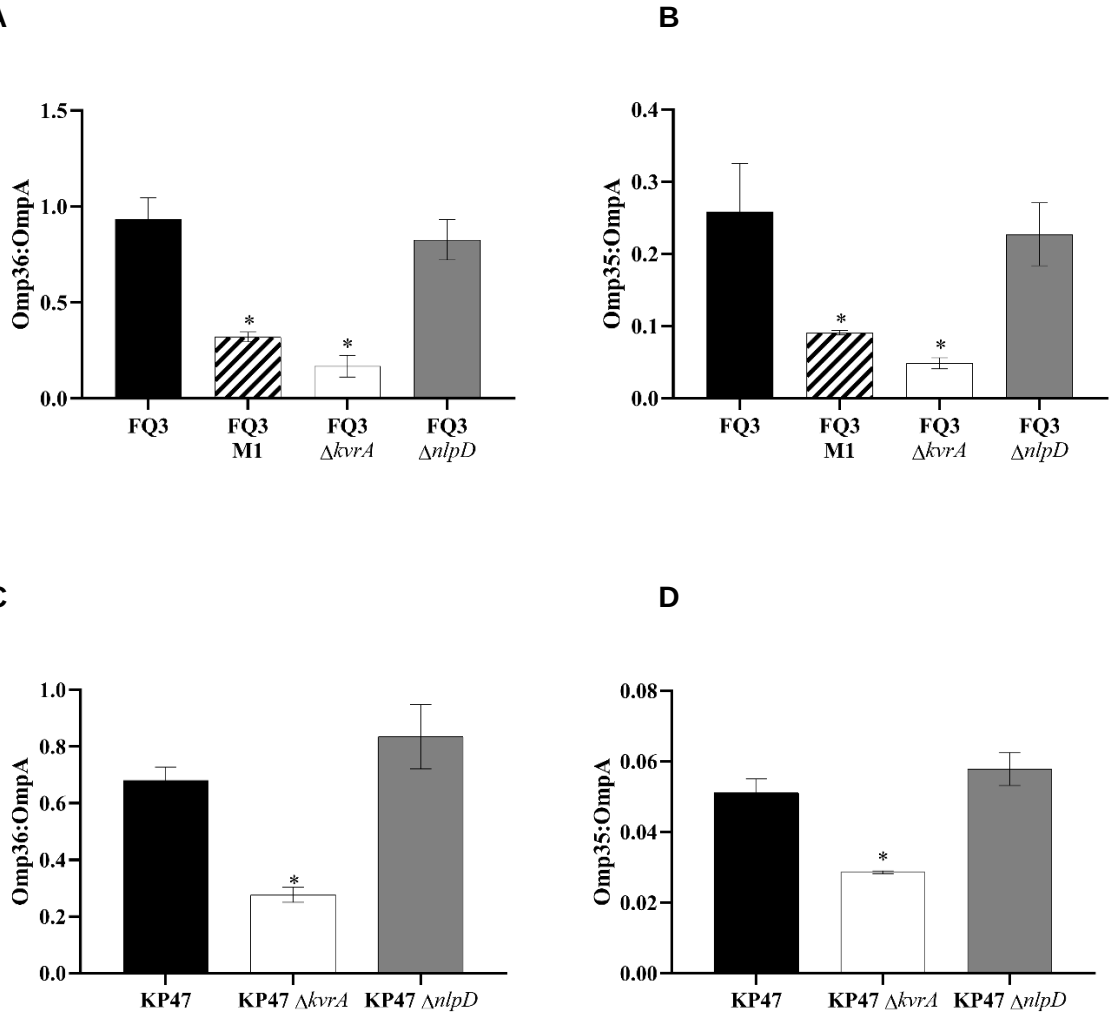
The CLSI susceptible and resistance breakpoints (11) for cefuroxime and cefoxitin are ≤8 and ≥32 µg.mL^{-1}. Values are modes of three repetitions.

Table 4. MICs (μg.mL^{-1}) of meropenem against *K. pneumoniae* clinical isolate KP47 and mutant derivatives measured with and without 8 μg.mL^{-1} of vaborbactam.

	Meropenem MIC	**Meropenem/Vaborbactam MIC**
KP47/pKPC-3	128	0.0625/8
KP47 Δ*ompK36*/pKPC-3	>256	32/8
KP47 Δ*nlpD*/pKPC-3	128	0.25/8
KP47 Δ*kvrA*/pKPC-3	256	1/8
FQ3/pKPC-3	128	0.125
FQ3 Δ*nlpD* /pKPC-3	256	0.125
FQ3 Δ*kvrA*/pKPC-3	>256	1

The CLSI susceptible and resistance breakpoints (11) for meropenem are ≤1 and ≥4 μg.mL^{-1} in the absence and ≤4 and ≥16 μg.mL^{-1} in the presence of 8 μg.mL^{-1} vaborbactam. Values are modes of three repetitions

Figure 1


A
B
Omp36:OmpA
Omp35:OmpA
1.5
1.0
0.5
0.0
0.4
0.3
0.2
0.1
*
FQ3
FQ3 M1
FQ3 ΔkvrA
FQ3 ΔnlpD
C
D
KP47
KP47 ΔkvrA
KP47 ΔnlpD
0.8
0.6
0.08
0.06
0.04
0.02
0.00

References

1. Grundmann H, Glasner C, Albiger B, Aanensen DM, Tomlinson CT, Andrasević AT, Cantón R, Carmeli Y, Friedrich AW, Giske CG, Glupczynski Y, Gniadkowski M, Livermore DM, Nordmann P, Poirel L, Rossolini GM, Seifert H, Vatopoulos A, Walsh T, Woodford N, Monnet DL. 2017. Occurrence of carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* in the European survey of carbapenemase-producing Enterobacteriaceae (EuSCAPE): a prospective, multinational study. Lancet Infect Dis 17:153–163.
2. Walsh TR. 2010. Emerging carbapenemases: a global perspective. Int J Antimicrob Agents 36:S8–14.
3. Queenan AM, Bush K. Carbapenemases: the versatile β-lactamases. 2007. Clin Microbiol Rev 20:440–458.
4. Lee C-H, Chu C, Liu J-W, Chen Y-S, Chiu C-J, Su L-H. 2007. Collateral damage of flomoxef therapy: in vivo development of porin deficiency and acquisition of bla_{DHA-1} leading to ertapenem resistance in a clinical isolate of *Klebsiella pneumoniae* producing CTX-M-3 and SHV-5 β-lactamases. J Antimicrob Chemother 60:410–413.
5. Nordmann P, Dortet L, Poirel L. 2012. Carbapenem resistance in Enterobacteriaceae: here is the storm! Trends Mol Med 18:263–272.
6. Pitout JDD, Nordmann P, Poirel L. 2015. Carbapenemase-Producing *Klebsiella pneumoniae*, a Key Pathogen Set for Global Nosocomial Dominance. Antimicrob Agents Chemother 59:5873–5884.
7. Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA. 2011. Carbapenems: past, present, and future. Antimicrob Agents Chemother 55:4943–4960.
8. Yan J, Pu S, Jia X, Xu X, Yang S, Shi J, Sun S, Zhang L. 2017. Multidrug Resistance Mechanisms of Carbapenem Resistant *Klebsiella pneumoniae* Strains Isolated in Chongqing, China. Ann Lab Med 37:398–407.
9. Wan Nur Ismah WAK, Takebayashi Y, Findlay J, Heesom KJ, Avison MB. 2018. Impact of OqxR loss of function on the envelope proteome of *Klebsiella pneumoniae* and susceptibility to antimicrobials. J Antimicrob Chemother 73:2990-2996.
10. Clinical and Laboratory Standards Institute. 2006. M2-A9. Performance standards for antimicrobial disc susceptibility tests; approved standard, 9th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
11. Clinical and Laboratory Standards Institute. 2019. M100-S29. Performance standards for antimicrobial susceptibility testing; twenty-ninth informational supplement. An informational supplement for global application developed through the Clinical and Laboratory Standards Institute consensus process. Clinical and Laboratory Standards Institute, Wayne, PA.

12. Jiménez-Castellanos JC, Wan Nur Ismah WAK, Takebayashi Y, Findlay J, Schneiders T, Heesom KJ, Avison MB. 2018. Envelope proteome changes driven by RamA overproduction in *Klebsiella pneumoniae* that enhance acquired β-lactam resistance. J Antimicrob Chemother 73:88-94.
13. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120.
14. Darling AE, Mau B, Perna NT, Batzoglou S, Zhong Y. 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One 5:e11147.
15. Cai SJ, Inouye M. 2002. EnvZ-OmpR interaction and osmoregulation in *Escherichia coli*. J Biol Chem 277:24155–24161.
16. Lange R, Hengge-Aronis R. 1994. The *nlpD* gene is located in an operon with *rpoS* on the *Escherichia coli* chromosome and encodes a novel lipoprotein with a potential function in ceil wall formation. Mol Microbiol 13:733–743.
17. Ichikawa JK, Li C, Fu J, Clarke S. 1994. A gene at 59 minutes on the *Escherichia coli* chromosome encodes a lipoprotein with unusual amino acid repeat sequences. J Bacteriol 176:1630–1638.
18. Tsang M-J, Yakhnina AA, Bernhardt TG. 2017. NlpD links cell wall remodeling and outer membrane invagination during cytokinesis in *Escherichia coli*. PLOS Genet 13:e1006888.
19. Martins-Pinheiro M, Lima WC, Asif H, Oller CA, Menck CFM. 2016. Evolutionary and Functional Relationships of the *dha* Regulon by Genomic Context Analysis. PLoS One 11:e0150772.
20. Zheng P, Wereath K, Sun J, van den Heuvel J, Zeng A-P. 2006. Overexpression of genes of the *dha* regulon and its effects on cell growth, glycerol fermentation to 1,3-propanediol and plasmid stability in *Klebsiella pneumoniae*. Process Biochem 41:2160–2169.
21. Palacios M, Miner TA, Frederick DR, Sepulveda VE, Quinn JD, Walker KA, Miller VL. 2018. Identification of Two Regulators of Virulence That Are Conserved in *Klebsiella pneumoniae* Classical and Hypervirulent Strains. MBio 9. pii:e01443-18.
22. Dorman MJ, Feltwell T, Goulding DA, Parkhill J, Short FL. 2018. The Capsule Regulatory Network of *Klebsiella pneumoniae* Defined by density-TraDISort. MBio. 9. pii:e01863-18.
23. Walker KA, Miner TA, Palacios M, Trzilova D, Frederick DR, Broberg CA, Sepúlveda VE, Quinn JD, Miller VL. 2019. A *Klebsiella pneumoniae* Regulatory Mutant Has Reduced Capsule Expression but Retains Hypermucoviscosity. MBio 10. pii:e00089-19.
24. Alexeyev MF. 1999. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. BioTechniques 26:824-828.
25. Clinical and Laboratory Standards Institute. 2015. M07-A10. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 10th

ed. Clinical and Laboratory Standards Institute, Wayne, PA.
26. Takebayashi Y, Wan Nur Ismah WHK, Findlay J, Heesom KJ, Zhang J, Williams OM, MacGowan AP, Avison MB. 2017. Prediction of cephalosporin and carbapenem susceptibility in multi-drug resistant Gram-negative bacteria using liquid chromatography-tandem mass spectrometry. BioRxiv 138594; doi: https://doi.org/10.1101/138594.
27. Wan Nur Ismah WAK, Takebayashi Y, Findlay J, Heesom KJ, Jiménez-Castellanos JC, Zhang J, Graham L, Bowker K, Williams OM, MacGowan AP, Avison MB. 2018. Prediction of Fluoroquinolone Susceptibility Directly from Whole-Genome Sequence Data by Using Liquid Chromatography-Tandem Mass Spectrometry To Identify Mutant Genotypes. Antimicrob Agents Chemother 62 pii:e01814-17.
28. Drawz SM, Bonomo RA. 2010. Three decades of β-lactamase inhibitors. Clin Microbiol Rev 23:160–201.
29. Hecker SJ, Reddy KR, Totrov M, Hirst GC, Lomovskaya O, Griffith DC, King P, Tsivkovski R, Sun D, Sabet M, Tarazi Z, Clifton MC, Atkins K, Raymond A, Potts KT, Abendroth J, Boyer SH, Loutit JS, Morgan EE, Durso S, Dudley MN. 2015. Discovery of a Cyclic Boronic Acid β-Lactamase Inhibitor (RPX7009) with Utility vs Class A Serine Carbapenemases. J Med Chem 8: 3682–3692.
30. Zhanel GG, Lawrence CK, Adam H, Schweizer F, Zelenitsky S, Zhanel M, Lagacé-Wiens PRS, Walkty A, Denisuik A, Golden A, Gin AS, Hoban DJ, Lynch JP, Karlowsky JA. 2018. Imipenem–Relebactam and Meropenem–Vaborbactam: Two Novel Carbapenem-β-Lactamase Inhibitor Combinations. Drugs 78:65–98.
31. Hackel MA, Lomovskaya O, Dudley MN, Karlowsky JA, Sahm DF. 2018. In Vitro Activity of Meropenem-Vaborbactam against Clinical Isolates of KPC-Positive Enterobacteriaceae. Antimicrob Agents Chemother 62:e01904-17.
32. Christina S, Jorgensen J, Rybak MJ. 2018. Meropenem and Vaborbactam: Stepping up the Battle against Carbapenem-resistant Enterobacteriaceae. Pharmacotherapy 38:444–461.
33. Lomovskaya O, Sun D, Rubio-Aparicio D, Nelson K, Tsivkovski R, Griffith DC, Dudley MN. 2017. Vaborbactam: Spectrum of β-Lactamase Inhibition and Impact of Resistance Mechanisms on Activity in Enterobacteriaceae. Antimicrob Agents Chemother 61:e01443-17.
34. Findlay J, Hamouda A, Dancer SJJ, Amyes SGB. 2012. Rapid acquisition of decreased carbapenem susceptibility in a strain of *Klebsiella pneumoniae* arising during meropenem therapy. Clin Microbiol Infect 18:140–146.
35. Tidhar A, Flashner Y, Cohen S, Levi Y, Zauberman A, Gur D, Aftalion M, Elhanany E, Zvi A, Shafferman A, Mamroud E. 2009. The NlpD lipoprotein is a novel *Yersinia pestis* virulence factor essential for the development of plague. PLoS One. 4:e7023.

133 36. Uehara T, Dinh T, Bernhardt TG. 2009. LytM-domain factors are required for daughter cell
134 separation and rapid ampicillin-induced lysis in *Escherichia coli*. J Bacteriol. 191:5094-
135 5107.
136 37. Uehara T, Parzych KR, Dinh T, Bernhardt TG. 2010. Daughter cell separation is controlled
137 by cytokinetic ring-activated cell wall hydrolysis. EMBO J. 29:1412-1422.
138 38. Dupont H, Choinier P, Roche D, Adiba S, Sookdeb M, Branger C, Denamur E, Mammeri
139 H. 2017. Structural Alteration of OmpR as a Source of Ertapenem Resistance in a CTX-
140 M-15-Producing *Escherichia coli* O25b:H4 Sequence Type 131 Clinical Isolate. Antimicrob
141 Agents Chemother. 61. pii:e00014-17.
142 39. Curran TD, Abacha F, Hibberd SP, Rolfe MD, Lacey MM, Green J. 2017. Identification of
143 new members of the *Escherichia coli* K-12 MG1655 SlyA regulon. Microbiology 163:400-
144 409.
145 40. Colgan AM, Kröger C, Diard M, Hardt WD, Puente JL, Sivasankaran SK, Hokamp K,
146 Hinton JC. 2016. The Impact of 18 Ancestral and Horizontally-Acquired Regulatory
147 Proteins upon the Transcriptome and sRNA Landscape of *Salmonella enterica* serovar
148 Typhimurium. PLoS Genet. 12:e1006258.
149 41. De la Cruz MA, Calva E. 2010. The complexities of porin genetic regulation. J Mol Microbiol
150 Biotechnol 18:24-36.
151 42. Liu P, Li P, Jiang X, Bi D, Xie Y, Tai C, Deng Z, Rajakumar K, Ou HY. 2012. Complete
152 genome sequence of *Klebsiella pneumoniae* subsp. *pneumoniae* HS11286, a multidrug-
153 resistant strain isolated from human sputum. J Bacteriol 194:1841-1842.
154 43. Bi D, Jiang X, Sheng ZK, Ngmenterebo D, Tai C, Wang M, Deng Z, Rajakumar K, Ou HY.
155 2015. Mapping the resistance-associated mobilome of a carbapenem-resistant *Klebsiella*
156 *pneumoniae* strain reveals insights into factors shaping these regions and facilitates
157 generation of a 'resistance-disarmed' model organism. J Antimicrob Chemother. 70:2770-
158 2774.